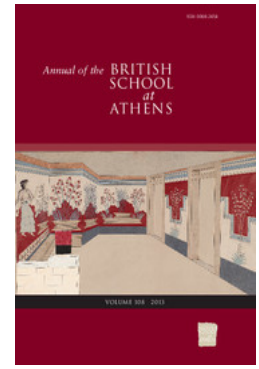
Kinship in Aegean Prehistory? Ancient DNA in Human Bones from Mainland Greece and Crete

Bouwman, Abigail S.; Brown, Keri A.; Brown, Terence A.; Chilvers, Elizabeth R.; Arnott, Robert; Prag, A.J.N.W.

DOI:
10.1017/S0068245400000265

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (Harvard):
Bouwman, AS, Brown, KA, Brown, TA, Chilvers, ER, Arnott, R & Prag, AJNW 2009, 'Kinship in Aegean Prehistory? Ancient DNA in Human Bones from Mainland Greece and Crete', *Annual of the British School at Athens*, vol. 104, pp. 293-309. https://doi.org/10.1017/S0068245400000265



Kinship in Aegean Prehistory? Ancient DNA in Human Bones from Mainland Greece and Crete

Abigail S. Bouwman, Keri A. Brown, Terence A. Brown, Elizabeth R. Chilvers, Robert Arnott and A.J.N.W. Prag

The Annual of the British School at Athens / Volume 104 / November 2009, pp 293 - 309
DOI: 10.1017/S0068245400000265, Published online: 10 June 2011

KINSHIP IN AEGEAN PREHISTORY? ANCIENT DNA IN HUMAN BONES FROM MAINLAND GREECE AND CRETE[1]

INTRODUCTION

In the *Annual* for 2000 we reported on a pilot project in which we had—successfully—searched for traces of ancient DNA (aDNA) in the skeletal remains from Grave Circle B at Mycenae, as part of a cross-disciplinary project seeking to discover links of kinship between the different groups of graves in which we used the methods of facial reconstruction and of epigenetic variation as well as DNA analysis (Brown *et al.* 2000). We believed that such an approach might cast light on dynastic links in a period of prehistory where there could be no texts to tell the story, and we ended that report on an optimistic note: advances in molecular biology and the concomitant development of techniques for studying aDNA made it likely that as well as establishing kinship relationships between burials or groups of burials (Brown 2001), in conjunction with bone isotope studies (Price 1989) it would be possible to identify individuals who might be incomers to a particular site. This would be especially interesting if applied to sites such as the Grave Circles at Mycenae, where the remains are presumed to be those of individuals who held high status in their societies, and whose family relationships might reveal the underlying processes by which such status was acquired.

Broader population studies might indicate affinities between the people living in different areas at different periods, possibly throwing light on questions such as the relationship between the Minoan and Mycenaean civilizations. Ancient DNA research could also answer long-standing questions about disease in the prehistoric Aegean, in particular to test hypotheses regarding the prevalence of malaria (Angel 1966). As well as searching directly for aDNA signatures of the malaria parasite in human bones (Sallares and Gomzi 2001), typing of globin gene mutations could determine if the skeletal indications of anaemia are indeed due to genetic thalassaemia rather the result of dietary iron deficiency (Chilvers 2004, where the rationale for this approach is explained).

Progress in any of these areas is clearly dependent on the survival of aDNA in human skeletons from sites in the eastern Mediterranean, in particular in Greece and the Aegean. It has become clear that the most important consideration in this regard is not the chronological age of the specimens but their thermal history, as DNA degradation occurs

[1] As ever we thank Helen Clark at the BSA for continued advice and guidance, and especially for assistance with obtaining permits. We also thank K.A. Wardle (University of Birmingham) for bone samples from Nea Nicomedia, M. Wiencke (American School of Classical Studies, Athens) for material from Lerna, S. Chryssoulaki (Ministry of Culture, Athens) for material from Karaviádena, M.Ph. Papakonstantinou (Ephorate of Prehistoric and Classical Antiquities, Lamia) for samples from Antron, L. Papazoglou-Manioudaki (National Archaeological Museum, Athens) for the Mycenae Grave Circle A remains, E. Palaiologou and her colleagues (Ephorate of Prehistoric and Classical Antiquities, Nafplion) for material from Mycenae Grave Circle B, W. Cavanagh (University of Nottingham) and C.B. Mee (University of Liverpool) for bones from Kouphovouno, and Anna Lagia and Josette Renard (Université de Montpellier) for help with the Kouphovouno material. We also thank M. Collins (York) for help with calculation of thermal ages and E.B. French for help, advice and encouragement, and Ian Barnes (University College London) and Laura Preston (University of Cambridge) for their perceptive and helpful comments as the *Annual*'s referees. The work was funded by the Institute for Aegean Prehistory, the Wellcome Trust and the Leverhulme Trust. Reports focussing on the scientific aspects of the work described here have also been published (Bouwman *et al.* 2008, Chilvers *et al.* 2008).

more rapidly at higher temperatures. A useful measure is 'thermal age' (Smith *et al.* 2003), which is calculated from the temperature history of a site and its geographical location. From experimental studies of DNA breakdown, together with a consideration of the oldest authenticated detections of aDNA, it has been estimated that the limit for DNA preservation is approximately 19,000 years at 10°C: hence specimens from any site that has a thermal age normalized to 10°C at >19,000 years are unlikely to contain aDNA. When the formula described in Appendix B of Smith *et al.* (2003) is applied, using modern data for mean annual temperatures and assuming that while *in situ* specimens were not subjected to substantial seasonal fluctuations, then for the eastern Mediterranean a thermal age of 19,000 years at 10°C corresponds to approximately 3600 chronological years (Chilvers 2004). This makes it unlikely that aDNA will be present at Neolithic and Early Bronze Age sites which predate 2000 BC, and suggests that the Bronze Age sites of the Minoan palace period (*c.*1850–1200 BC) and of Mycenaean Greece (*c.*1650–1150 BC) will be at the very limits for aDNA preservation. At these sites we can therefore anticipate that local factors such as exposure to water and the time that has elapsed since excavation (Pruvost *et al.* 2007) will be crucial in determining if aDNA can be recovered.

This article presents the next stage in our research into aDNA: whereas the pilot was restricted to material from Grave Circle B, we have extended it both in time and space in order to work with a wider corpus of samples. The sites from which we have been able to take samples are Nea Nikomedia, Lerna, Karaviádena (Zakro), Antron (Fthiotida), Kouphovouno, and Mycenae, ranging in date from the later seventh millennium BC to the middle of the second and covering a geographical spread from central Macedonia to Laconia and on to eastern Crete. The timing of our work was such that through the kindness of Dr Lena Papazoglou-Manioudaki we were able in 2006 to sample the two recently rediscovered skeletons from Shaft Grave VI at Mycenae, and through collaboration with Professor William Cavanagh we were also able to study material excavated between 2001 and 2002 at Kouphovouno. In parallel to the DNA studies, during 2007–8 our colleague Dr Argyro Nafplioti carried out strontium isotope analyses of the human remains from both grave circles at Mycenae (Nafplioti 2009).

Previous studies of bones and teeth from prehistoric Greece support the prediction that aDNA is recoverable from some sites dating to 2000 BC, with local factors having an important impact on preservation (Evison *et al.* 1999; Brown *et al.* 2000; Evison 2001). Although carried out to the highest standards prevailing at the time, these studies took place before stringent criteria for aDNA authenticity had been established (Cooper and Poinar 2000), and in retrospect it appears probable that some of the 'detections' of aDNA described in these papers were in fact due to modern contamination. Conversely, it is possible that some specimens that gave negative results in these projects would have yielded authentic aDNA if the highly sensitive polymerase chain reactions (PCRs) now available had been used. As well as continuing our work at Mycenae, we have therefore also carried out a systematic survey of aDNA preservation at six Neolithic and Bronze Age sites in Greece and Crete, examining 89 skeletons in total, and using the most up-to-date aDNA techniques, in order to evaluate the broader potential of aDNA research in the Eastern Mediterranean.

Materials and Methods

1. Sites and bone specimens

Bone specimens are listed in TABLE 1 and the locations of the sites from which these were obtained are shown in FIG. 1. The early Neolithic village of Nea Nikomedia is a multi-period settlement mound located on the central Macedonian plain, close to the south-west border of Lake Ludias marshland and excavated in 1961–4 (Rodden 1962; Wardle 1996; Angel 1973*a*). Calibrated radiocarbon dates for the earliest phases of occupation at the site fall into the range 6400–6000 BC (Perles 2001, 99–110). In contrast with other early Neolithic sites, burials have been found within the settlement, many in shallow, irregular pits located outside the houses or in the rubble of older collapsed houses rather than under the floors (Perles 2001, 276–9). Lerna is located in the south-east Argolid, on the western shore of the Bay of Argos and was occupied from the sixth to the first millennia BC; excavations were carried out between 1952 and 1958. During the first phases of occupation in the Neolithic it is likely that the site was some distance from the sea, but as sea levels continued to rise, significant regions of the Argive plain were covered with water and Lake Lerna was formed, creating a marshy environment in the vicinity of the site (Zangger 1991). Although the Neolithic contexts yielded ten burials (Angel 1971, 39–41), the majority date from the Middle Bronze Age (2050/2000–1700/1675 BC). There are no burials in the Early Bronze Age contexts and later burials are rare. The Middle Bronze Age burials are usually found either next to the houses

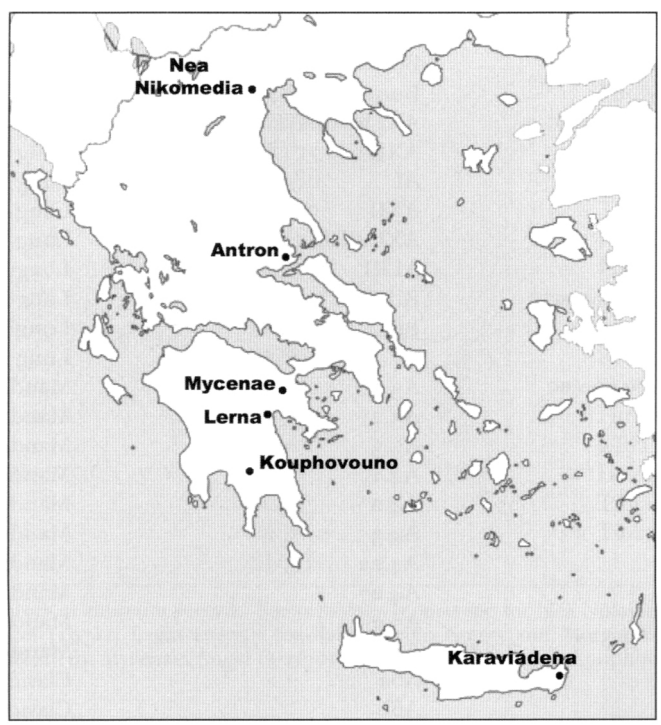


FIG. 1. Locations of sites.

TABLE 1. Bone specimens.

Site	Date	Number[a]	Description
Nea Nikomedia	6400–6000 BC	Infant 1 (A6/1)	Long bone fragment
		Infant 3 (Ω6/1A)	Long bone fragment
		Infant 5 (B6/2)	Long bone fragment
		NN4 (NN XLI D5/1 IV)	Left clavicle fragment
		NN13* (NN XIIIR7/1C)	Short rib fragment
		NN19 (NN XIX C9/1d)	Long bone fragment
		NN5 (NN L E3 V)	Second metatarsal
		NN23 (NN XXIII TX–5/1)	Right second metatarsal
		NN28 (XXVIII Tα2)	Left third metatarsal
Lerna	6th millennium BC	Ler 220 (EN)	Mandible fragment
	2050–1675 BC	Ler 10	Cranial fragment
		Ler 103	Long bone fragment
		Ler 81	Radius fragment
		Ler 125	Cranial fragment
		Ler 48	Patella
		Ler 203	Cranial fragment
		Ler 72	Radius fragment
Karaviádena	2000–1700 BC	Burial 1a (A1-A2-K3, ZK15)	Left talus
		Burial 1b (A1-A2-K3, ZK16)	Left talus
		Burial 1c (A1-A2-K3, ZK17)	Left talus
		Burial 1d (A1-A2-K3, ZK18)	Left talus
		Burial 2 (A3, ZK8)	Long bone fragment
		Burial 3 (A4-A7, ZK10)	Long bone fragment
		Burial 4–5 (K1-A-A5, ZK19)	Long bone fragment
Antron	2000–1700 BC	AXLVIIIbMH	Skull fragment
Grave Circle A		AXII	Long bone fragment
		AI	Long bone fragment
		AXII	Long bone fragment
		AXLVIIIbLH	Long bone fragment
		ALIII	Long bone fragment
Antron	2000–1700 BC	BII	Long bone fragment
Grave Circle B		BIII	Long bone fragment
		BV	Long bone fragment
Mycenae	1600–1500 BC	A3.1	Mandible
Grave Circle A		A3.2	Mandible
		A3.3	Mandible
		A4.1	Mandible
		A4.2	Mandible
		A4.3	Mandible
		A4.22	Mandible
		A4.27	Mandible
		A5.25	Mandible
		A5.26	Mandible
		A6a	Clavicle
		A6b	Clavicle
		AM	Unrecorded

Site	Date	Number[a]	Description
Mycenae	1650–1550 BC	Γ51	Long bone fragment
Grave Circle B		B52	Long bone fragment
		P53	Long bone fragment
		H54	Long bone fragment
		Γ55	Long bone fragment
		Λ56	Long bone fragment
		Ξ57	Long bone fragment
		Γ58	Long bone fragment
		Z59	Long bone fragment
		Δ60	Long bone fragment
		Δ61	Long bone fragment
		A62	Long bone fragment
		Θ63	Long bone fragment
		N66	Long bone fragment
		N66a	Long bone fragment
		I68	Long bone fragment
		A69	Long bone fragment
		K70	Long bone fragment
		Λ70a	Long bone fragment
		Λ70a1	Long bone fragment
		Λ70a2	Long bone fragment
		Λ70a3	Long bone fragment
Kouphovouno	2000–1700 BC	KE009	Tibia fragment
		KE009B	Tibia fragment
		KE105	Humerus fragment
		KE108	Radius fragment
		KE171	Radius fragment
		KE173	Humerus fragment
		KE186	Tibia fragment
		KE207	Tibia fragment
		KE213L	Humerus fragment
		KE213U	Fibula fragment
		KE216	Rib fragment
		KE220	Femur fragment
		KE601	Femur fragment
		KE704	Skull fragment
		KE705	Humerus fragment
		KE706	Ulna fragment
		KE707	Humerus fragment
		KE713	Tibia fragment
		KE715	Tibia fragment
		KE716	

[a] According to site, inventory or museum records. For osteology reports see for Nea Nikomedia: Angel (1973a), Lagia (1993); for Lerna: Angel (1971), Lagia (1993); for Karaviádena: Arnott and Morgan-Forster (in press); Antron: A.Papathanasiou (unpublished); Mycenae: Angel (1973b), Musgrave *et al.* (1995); Kouphovouno: Lagia (in Cavanagh *et al.* 2007).

or, in the case of some of the infant burials, under the floors of houses. The site of Karaviádena is located on the eastern coast of Crete, somewhat less than a kilometre south of the great Middle-Late Minoan palace at Zakro. During the construction of a road from Epano Zakro to Kato Zakro a grave containing what appear to be six burials was discovered here, and subsequently excavated in 1994 by the Greek Archaeological Service. The burials are believed to date from Middle Minoan II phases of occupation in the area (1850–1700 BC) and may form part of a larger cemetery (at present unpublished, but see Arnott and Morgan-Forster in press; Arnott and Stuckey 2003). Antron (Glypha Bay, Fthiotida) is located on the east coast of mainland Greece. Two grave circles (A and B) adjacent to one another were excavated in 1990–1995: most of the burials were in cist graves and dated to the Middle Helladic III to Late Helladic II A periods (1750–1450 BC) (Papakonstantinou 1999*a* and *b*). Mycenae is located in the north-east of the Peloponnese. Grave Circles A and B date to 1675–1500 BC, Grave Circle B predating A with possibly fifty years' overlap between the two. The Grave Circles therefore date to the very beginning of the Mycenaean age at the boundary of the Middle to Late Helladic periods. Within Grave Circle B, excavated in 1952–4, there is a development from simple cist burials to larger, deeper and richer Shaft Graves, while Grave Circle A, dug in 1876–7, comprises six Shaft Graves (Mylonas 1957). Kouphovouno, located in Laconia just south of Sparta, spans the Middle Neolithic to Late Bronze Age periods (*c.*5000–1200 BC), and was the subject of a major excavation by the British School during 2001–2005. Twenty-seven burials were recovered, most of them from a Middle Bronze Age cemetery (2000–1700 BC) and mostly from shallow earth graves (Cavanagh and Lagia, forthcoming; Cavanagh *et al.* 2007; Lagia *et al.* 2007).

2. DNA TECHNIQUES

The techniques used to extract the DNA and the regime followed in analysing the results are described in detail in Bouwman *et al.* (2008) and Chilvers *et al.* (2008). Briefly, surface contamination, including DNA deposited on the bones by excavators and curators, was reduced by removing the outer 1–2 mm of each bone sample with a sterile scalpel and irradiating with UV. Approximately 0.5 g was then removed from the core of each bone and any DNA present in the sample extracted by soaking the powder in a buffer. The DNA was then concentrated and an aliquot tested using a series of PCRs (up to 34 for each specimen) designed to amplify diagnostic regions of the mitochondrial and nuclear genomes. The mitochondrial loci that were studied were the ones containing mutations that enable mitochondrial haplogroups to be assigned, these haplogroups revealing possible maternal relationships as the mitochondrial DNA (mtDNA) is inherited solely through the female line. The nuclear loci included many of the variable sites typed by forensic scientists in order to construct genetic profiles from which both maternal and paternal relationships can be inferred.

3. ANCIENT DNA REGIME

We carried out the work in accordance with the standard criteria of authenticity for aDNA research (Cooper and Poinar 2000) as far as was possible. To avoid cross-contamination with DNA from previous experiments, extractions and PCRs for the Nea Nikomedia, Lerna, and Karaviádena specimens were set up in laminar flow cabinets in physically isolated labs, and those for the Antron, Mycenae and Kouphovouno specimens were set up in similar labs but

each with an ultrafiltered air supply, with specimens handled and extractions prepared within a biological safety cabinet, and PCRs set up within a laminar flow hood. All extractions were accompanied by negative controls in which the entire extraction procedure was performed without bone material, and all PCRs were accompanied by negative controls containing water instead of DNA extract. Ancient DNA molecules become broken into fragments during diagenesis and hence are shorter than modern contaminants, and therefore the lengths of the template molecules in all extracts giving positive PCRs were assessed to ensure that they fell in the anticipated range. To confirm the identity of a PCR product, the DNA was cloned before sequencing, as this procedure enables mixed products (e.g. specimens containing both aDNA and modern contaminants) to be identified, and also enables aDNA sequences to be recognised by virtue of the chemical damage they have undergone during diagenesis. Because of the small amounts of material that were available, it was not possible to carry out some other checks that ideally would have been performed. It was not possible to perform replicate extractions for all skeletons, nor was it possible to divide the bone samples so that portions could be sent to a second lab for independent testing, and, similarly, there was insufficient material to carry out tests aimed at determining the overall level of biomolecular preservation in the specimens, such as measurements of collagen content. Corroboration of the human results could not be sought through study of associated animal remains, as no animal remains were available. As mentioned above, we removed the outer 1–2 mm of each bone prior to preparation of extracts. We have shown that even after extensive handling most of the contaminating DNA in a bone resides in the outer 1–2 mm (Bouwman *et al.* 2006), and that very little redistribution occurs if the bone is washed as in standard archaeological practice (M.M. Mundee, A.S. Bowman and T.A. Brown, work in progress).

Results

The results are summarized in TABLE 2. No evidence of aDNA was obtained with any of the specimens from Nea Nicomedia, Lerna, Karaviádena, Antron Grave Circle A or Mycenae Grave Circle A. With all but one specimen from these sites, PCRs failed to give any products. The exception was sample ZK8 from Karaviádena, which gave products of the correct size with one of the mtDNA PCRs and with a PCR directed at a sex-identifying region of the nuclear DNA. However, the sequence of the mtDNA product was identical with that of E.R.Chilvers, who studied this specimen, and further examination showed that the DNA present was >425 bp, longer than most genuine aDNA molecules, even those from the best-preserved material (O'Donoghue *et al.* 1996). These results suggest that ZK8 had become contaminated with modern DNA from E.R.Chilvers. The possibility that aDNA was present in these bone extracts but undetectable due to the presence of co-purifying substances that were inhibitory to PCR was tested by 'spiking' PCRs of modern human DNA with bone extracts. These control PCRs was unaffected by addition of any bone extract, indicating that inhibitory substances were absent.

The results with the three specimens from Antron Grave Circle B were inconsistent but could possibly indicate the presence of aDNA. Although mtDNA could not be detected, two of the nuclear PCRs gave positive results with extracts of specimens BII and BIII, and a range of positive results were obtained after nuclear PCRs with specimen BV. Replicate PCRs did not, however, give reproducible results and in general the yields of DNA were weak.

TABLE 2. Summary of results.

Site	PCRs attempted with each specimen[a]	Evidence for aDNA
Nea Nicomedia	MtD (2), MtH (2), GA (2)	None
Lerna	MtD (2), MtH (2), GA (2)	None
Karaviádena	MtD (2), MtH (2), GA (2)	None
Antron Grave Circle A	MtC (2), D2S11338 (2), D5S818, D10S1248, D14S1434, D16S539 (2), D18S51 (2), D22S1045, FGA (2), THO1 B (2), DYS426, M35, GA (2), MB (2)	None
Antron Grave Circle B	MtC (2), D2S11338 (2), D5S818, D10S1248, D14S1434, D16S539 (2), D18S51 (2), D22S1045, FGA (2), THO1 B (2), DYS426, M35, GA (2), MB (2)	No evidence of mtDNA. Inconsistent results for nuclear DNA in all three bones studied.
Mycenae Grave Circle A	MtA (2), MtG (2), MtC(2), MtF, MtD (2), MtW, MtV, CD4, D1S656, D2S1338, D3S1358 (2), D5S818, D6S366, D8S535, D8S1179 (2), D10S1248, D10S2325 (2), D14S1434, D16S539, D18S51, D22S1045, FGA, THO1 A (2), THO1 B, VWA (2), DYS389, DYS391, DYS393, DYS426 (2), DYS460 (2), M35, M173 (2), GA (2), MB	None
Mycenae Grave Circle B	MtA, MtG, MtC, MtF, MtD, MtW, MtV (2), CD4 (2), D1S656, D2S1338, D3S1358 (2), D5S818, D6S366, D8S535, D8S1179, D10S1248, D10S2325 (2), D14S1434, D16S539, D18S51, D22S1045, FGA, THO1 A, THO1 B, VWA (2), DYS389, DYS391, DYS393, DYS426, DYS460 (2), M35, M173, GA, MB	mtDNA in Γ55, Γ58, Z59 and A62.
Kouphovouno	MtC (3), D2S1338, D10S1248, D14S1434, D16S539, D18S51, D22S1045, FGA, THO1 B, GA (3), MB	mtDNA and/or nuclear DNA in 7 bones.

[a] Numbers in brackets indicate PCRs that were carried out more than once with each specimen.

Nuclear DNA was occasionally detected in specimens from Mycenae Grave Circle B, but too sporadically for the results to be authenticated. With PCRs directed at mtDNA, 18 of the 22 samples never gave a PCR product of the correct size, or if they did then that product was considered to be non-endogenous to the sample because it was accompanied by contaminated negative controls, was entirely made up of sequences containing an unusual mutation possessed by A.S. Bouwman, who performed all these extractions and PCRs (we assumed that every sequence containing this mutation was a contaminant derived from A.S. Bouwman), or was not human mtDNA. The other four samples (Γ55, Γ58, Z59, and A62) gave sequences which were considered to derive, at least in part, from ancient DNA. These results are described in detail in the final section of this paper.

The bones we studied from Kouphovouno were excavated during 2001–2 under conditions designed to minimize contamination with modern DNA, and the excavator and A.S.

Bouwman were the only people who handled these bones prior to transfer of samples to the high-containment laboratory at Manchester. The mitochondrial and nuclear DNA features of the excavator and A.S. Bouwman are known. It has been suggested that once all sequences identical to those of individuals who have handled a specimen are excluded, then any sequences that remain are likely to be genuine aDNA (Sampietro *et al.* 2006). On this basis, two of the Kouphovouno specimens (KE009B and KE105) contain mitochondrial aDNA, and six (KE009B, KE173, KE207, KE601, KE706, KE715) contain nuclear aDNA.

Discussion

Validation of aDNA research has been discussed extensively in the literature, with the 'criteria of authenticity' proposed by Cooper and Poinar (2000) considered by many to be the gold standard against which such work should be judged. Sometimes, however, these criteria are difficult to meet because of the realities of biomolecular archaeology, in particular the problems posed by the limited amount of material that is usually available for study. Museum curators are, understandably, unwilling to allow destructive analysis of anything more than very small samples taken from human specimens, and their reluctance is likely to become greater with the growing debate regarding 'ownership' of human archaeological remains. The requirements within the 'criteria of authenticity' for multiple extractions and PCRs to check reproducibility of results, replication of extractions and PCRs in a second lab, and analysis of specimens to assess the overall degree of biomolecular preservation, are reasonable if one is working with sufficient material but are not easy to satisfy if only a gram or so of bone is available. Recognising this problem, Gilbert *et al.* (2005) have recommended that biomolecular archaeologists take a cognitive and self-critical approach to authentication of results, which is what we attempt to do here.

A key component of a cognitive approach to authentication of aDNA detection is a consideration of the age and preservation conditions of the specimens under study and the time that has elapsed since their excavation, and an evaluation of whether these factors make it possible for DNA to have survived. As temperature is the primary determinant of the rate of DNA breakdown, the thermal history of a site can give an indication of the likelihood of aDNA presence in specimens, but such analyses are at best approximate due to difficulties in determining factors such as seasonal temperature fluctuations and the precise conditions in the microenvironment occupied by the buried specimens (Smith *et al.* 2003). However, assessment of the thermal history of a site gives an indication of the age beyond which specimens are unlikely to contain aDNA—placing a large burden of proof on researchers claiming aDNA detections with older material—and helps identify in which specimens aDNA survival is possible, providing a starting point for self-criticism of results. In this context, judgment of the authenticity of results at one site is aided by information on the extent of DNA survival at other sites within the same geographical region and hence likely to be of similar thermal ages. Our main focus has been on Grave Circle B at Mycenae, whose thermal age is right on the limit for aDNA preservation. To aid in assessment of the DNA detections that we made at Grave Circle B, we therefore surveyed aDNA survival at various other sites in Greece and Crete, from the Neolithic and Bronze Age, sites whose thermal histories also place them at the very limits of expected survival time for aDNA.

We found possible evidence for aDNA at three of the eight sites that we studied. At Antron

Grave Circle B we detected nuclear but not mitochondrial DNA in each of the three skeletons that we tested. These results were inconsistent with replicate PCRs failing to give reproducible results. The fact that only nuclear DNA could be detected is worrying, as mtDNA is present in a much higher copy number and hence rarely undetectable if genuine nuclear aDNA is present. On balance, we believe that these results are due to contamination of the bones with modern DNA from previous PCR experiments carried out in the laboratory. If the results do indicate the presence of aDNA in these specimens, then that aDNA is very poorly preserved and unlikely to yield useful information. At Mycenae Grave Circle B we obtained evidence for mitochondrial aDNA in four of the 22 skeletons that we studied. A full authentication of these results appears in the scientific report of our Mycenae study (Bouwman *et al.* 2008), and the archaeological implications of the aDNA sequences as the final section of this article. At Kouphovouno we also obtained evidence for aDNA that, subject to more detailed assessment, we believe to be genuine because the genetic features of the aDNA differ from those of the only two individuals who could have contaminated the bone samples.

Equally important are the negative results that we obtained. We have no evidence whatsoever of aDNA in specimens from Nea Nicomedia, Lerna, Karaviádena, or Mycenae Grave Circle A. For the specimens from Nea Nicomedia and for Lerna no. 220 this result is far from surprising because at 7000–8000 years these bones are substantially older than the expected limit (3600 years) for aDNA survival in Greece based on calculations of thermal age (Chilvers 2004). The younger specimens from Lerna are dated to 2050–1675 BC and hence closer to the 3600 year age limit, but the marshy conditions that have prevailed in the vicinity of Lerna for at least part of the period that these skeletons have been buried suggests a relatively high moisture content likely to promote DNA degradation. While these conditions rendered it more likely that the ancient inhabitants of Lerna suffered from malaria (and the bone evidence suggested that anaemia was common), it vitiated the hopes of being able to find the aDNA signatures of the malaria parasite and the globin gene mutations associated with genetic thalassaemia (Chilvers 2004).

The specimens from Karaviádena (2000–1700 BC) had previously been sampled in 2001 at Manchester by Elizabeth Chilvers (neé Stuckey) as part of Arnott's study of malaria in the prehistoric Aegean, and the negative results that we report here derive from that study (Arnott and Stuckey 2003; Arnott and Morgan-Forster in press). Both these bones and those from Mycenae Grave Circle A (1600–1500 BC) are close to the thermal age limit and hence possibly expected to show some indication of aDNA survival. However, those from Karaviádena were poorly preserved at the time of excavation, being highly fragmented, suggesting that overall biomolecular preservation might be poor. The Mycenae Grave Circle A bones, excavated in 1876–7, have been housed in museums for 130 years, and it is now clear that aDNA breakdown accelerates after excavation of bones (Pruvost *et al.* 2007): thus any aDNA present in the Mycenae bones when Schliemann and Stamatakis discovered them will almost certainly have degraded during the intervening decades.

We conclude that, although aDNA might be present in some skeletons from later centuries of the Greek Bronze Age, it is not commonly present in Greek material from this period and is likely to be absent from older material. In reaching this conclusion, we used optimized PCR systems in order to maximize our chances of detecting aDNA if it was present, but we also used an ultraclean facility and took scrupulous care to remove surface contamination from the bone samples, to prevent cross-contamination with PCR products from previous experiments,

and to identify contamination that remained. We also confirmed that negative results were not due to inhibition of PCRs by substances co-purifying with aDNA. We therefore believe that all putative detections of aDNA from the Neolithic and Bronze Age periods of Eastern Mediterranean prehistory require convincing authentication, whether through self-criticism over results or through adherence to criteria of authenticity.

Grave Circle B at Mycenae

Whether a group of skeletons buried in proximity to one another represents the members of a single family can be a key question when human remains are excavated at archaeological sites of any age. Our work at Mycenae was conceived as a search for kinship among the people buried in Grave Circle B. The graves in this circle appear to have been laid out in three groups in the south-east, north-west, and north-east sectors of the circle, with a fourth group comprising two graves (E and Γ) just east of the centre. They were named by the excavators with the letters of the Greek alphabet to distinguish them from those in Circle A which had been given Roman numerals; later Angel identified the individual skeletons with Arabic numerals in the order in which he studied them. Archaeologically one could only guess at what relationship, if any, the occupants of the four groups of graves might have to each other: did each group represent different families, or just different branches of the same family? Facial reconstruction had already identified three distinct facial types among the seven skulls that could be reconstructed, which we thought might represent different family groups (Prag *et al.* 1995): Γ55, Γ58, and A62 all had heart-shaped faces with wide-set cheekbones and eyes, and small, rather delicate features; Γ51 and Z59 had long faces with high foreheads, lantern jaws, and narrow features, while B52 had a large beaky face in a small head, and probably represents a third type or family. Finally, Σ131 had something in common with both the first two types. In terms of relative dating, these individuals covered the whole period of use of the grave circle (*c.*1675–1550 BC): Z59 and Σ131 were buried early in the circle's use, B 52 was 'early middle', and A 62 along with the three individuals in Grave Γ were all late. The layout of the graves and the kinship connections suggested by facial reconstruction are shown in FIG. 2.

Ancient DNA has been used to study such relationships in a historic context (Gill *et al.* 1994; Gerstenberger *et al.* 1999; Dudar *et al.* 2003; Gilbert *et al.* 2005), and so it was introduced here to test or to support the results suggested by facial reconstruction after a pilot project to confirm the survival of DNA in the bones (Brown *et al.* 2000). Altogether we tested 22 of the skeletons from Grave Circle B, including all those for which facial reconstructions had been carried out, except for Σ131: in this case it was no longer possible to identify the associated post-cranial bones in the Nauplia Museum and the skull itself was in too good condition to permit any intrusive sampling, so to our great regret it could not be tested for DNA. Our experiences with specimens from the other sites that we studied warned us that at best we could expect to detect DNA in only a few of these skeletons, and this turned out to be the case, with 18 of the Grave Circle B inhabitants giving entirely negative results. The four other skeletons yielded evidence for mitochondrial but not nuclear DNA. These four skeletons were Γ55, Γ58, Z59, and A62. The fact that facial reconstructions were available for each of these is perhaps not just a fortunate coincidence as the reconstructions had been performed on the best-preserved skulls, which one might expect to be from the skeletons

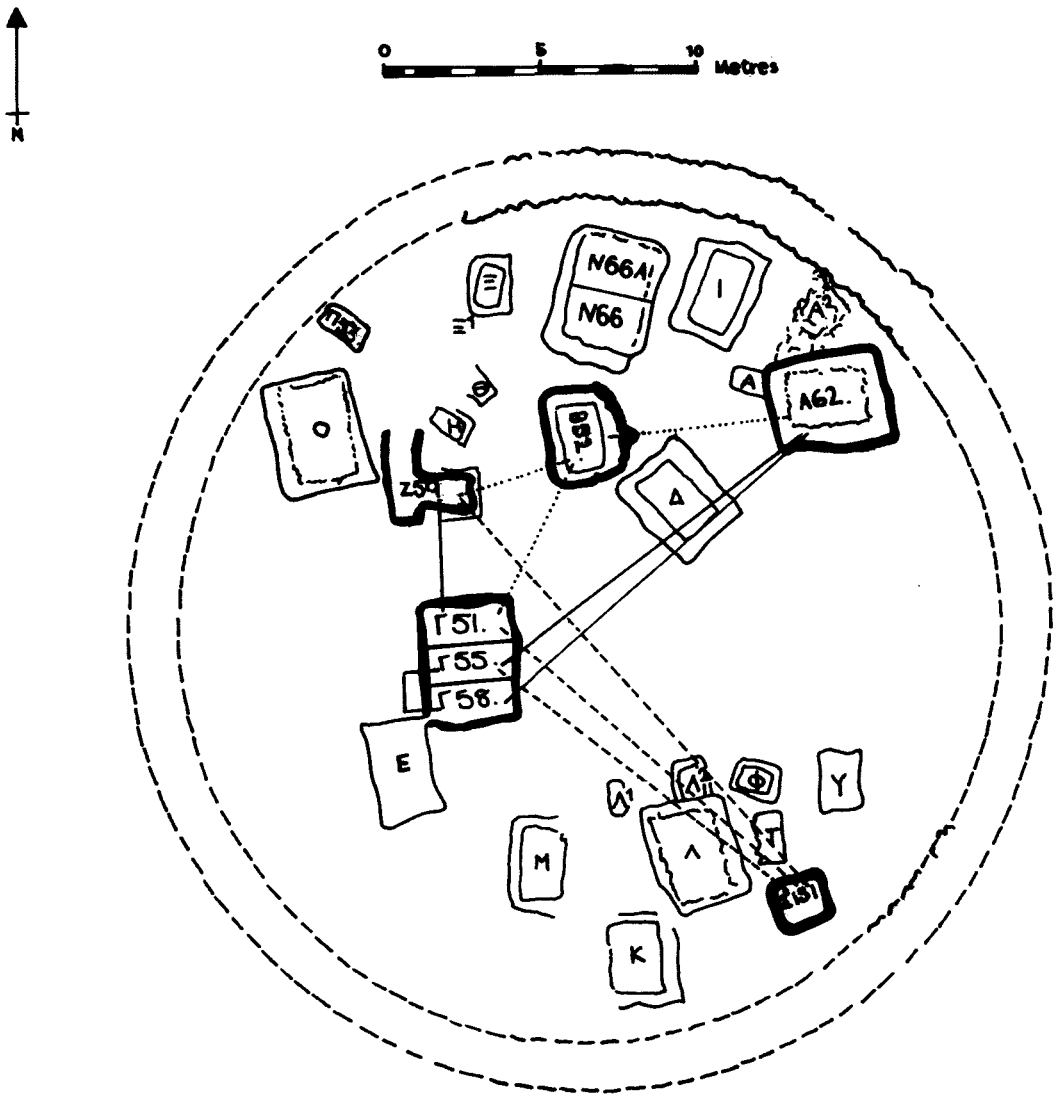


FIG. 2. Plan of Grave Circle B showing kinship connections between graves based on facial reconstructions. Solid lines indicate probable links, broken lines possible links, and dotted lines tentative links.

displaying the best overall preservation and hence the greatest likelihood of containing ancient DNA. The DNA sequences of two of these individuals, Γ55 and Γ58, were identical, but different from that of Z59. With A62 the DNA was very poorly preserved, but from the limited information that we could obtain we were able to establish that its sequence was different again, representing a third class. Why do we have confidence that these detections are of genuine aDNA and not modern contamination? First, there is the information gained from other sites suggesting that aDNA can, under favourable circumstances, survive in material from the Greek Bronze Age. Second, from the identity of the sequences we can

exclude the possibility that they derive from laboratory contamination that occurred after we took our samples. Of course, the bones had been handled prior to our sampling and contamination might have occurred then. Grave Circle B was excavated in 1952–4, using the procedures current at that time and hence without precautions to prevent DNA contamination. We established, however, that since excavation the bones had not been extensively handled, except by Dr J.L. Angel and his two assistants, who carried out the osteological examination in 1954. We therefore surmise that extensive contamination by multiple individuals is unlikely. The fact that three different DNA sequences were obtained from the four skeletons therefore becomes important. We argue that the DNA that we have identified could be modern contamination, if: (i) Γ55 and Γ58 were handled by one or more individuals who did not handle, or at least did not contaminate, any of the other 20 specimens that we studied; (ii) Z59 was handled by a different individual who did not contaminate any of the other 21 bones; and (iii) A62 was similarly handled by another different individual who did not contaminate any of the other 21 bones. This scenario is *possible* but we consider the alternative explanation, that these DNAs are ancient in origin, to be *more likely* (Bouwman *et al.* 2008).

As the tests only yielded data for the mitochondrial DNA, we could infer only the maternal relationships and nothing through the male line. Γ55 and Γ58 could share a maternal relationship. There seems little doubt that Γ58 is a woman: even before the DNA evidence, Angel had noted that although she was tall and strongly built, 'browridges likewise agree with the markedly female true pelvis (birth canal) and pelvis in showing female sex' (1973b, 381 and table 1). Interestingly, although Γ55 was identified as male by both Mylonas and Angel on the grounds of grave assemblage and the skeletal remains respectively, the first round of DNA analyses suggested that this might possibly be a female skeleton, although this was later rejected on the grounds that the female DNA results were less certain and the repeat test proved unsuccessful (Mylonas 1973, i. 46–7; Angel 1973*b*, 379–80 and table 1; Brown *et al.* 2000, 117 and table 1). Many of the graves in both circles contain multiple burials made over a period of time, and the sequence in Grave Γ seems to have been first an unidentified individual (probably male), then Γ58 after an interval long enough for the first skeleton to have become completely disarticulated, and finally Γ55 and Γ51 (Angel 1973*b*, 381; Mylonas 1973, i. 48–9). The fact that Γ58's skeleton was still well articulated suggests that she was buried only a few months before Γ55 and his companion Γ51; there is no evidence from any of the other burials to suggest the use of a shroud that would have kept her skeleton together after the connective tissue had decomposed.

Γ58 and first Γ55 were also close in age: Angel and Musgrave both reckon that he was probably around 33 and she was perhaps 36 years old at death (Angel 1973*b*, 379–81; Musgrave in Prag *et al.* 1995, 132–3). Therefore not mother and son; the simplest interpretation is that they were brother and sister, but they could equally be cousins whose mothers were sisters, or second cousins whose maternal grandmothers were sisters, and so on. It is of course possible that they are unrelated but just by chance have the same mitochondrial DNA, but as their particular DNA occurs in only approximately 5% of Europeans today, it is much more likely that they are related in one of the ways described.

The aDNA evidence tells us that Z59 does not have any maternal relationship with Γ55 or Γ58. He is not a full brother of Γ55 or Γ58 nor the son of Γ58. We cannot say anything about his paternal line. So, for example, he could in theory share a father with Γ55 and/or Γ58 but

have a different mother, he could be the father of one or both, or he could be Γ55's son by someone other than Γ58, but our data tell us nothing on this score. The facial evidence suggests a close relationship with Γ51, though the two men were buried in different parts of the grave circle and are maybe three generations apart, but there are no DNA results to help us. The fact that Γ51 was given a relatively poor burial next to two very rich people in this late grave may suggest a shift in family status and relationships.

The same conclusions apply for A62. He is the one for whom the DNA results are the least secure: according to the available DNA data he has no maternal relationship with Γ55, Γ58, or Z59, although he shares some facial features with Γ55 and Γ58 such as the widely set eyes and cheekbones and the angle of cheek to chin. He was probably in his mid-twenties when he died, a little earlier than Γ55 and Z59 and buried in another part of the Grave Circle. The ages at death and burial dates are too close for him to have been their father, but it is always possible that he was a paternal cousin. We have already suggested elsewhere that the central position of Grave Γ—probably the latest in the circle—suggests some kind of rapprochement or coming together of different branches of the family or of different families (Prag *et al.* 1995, 128–9; Prag and Neave 1999, 141–2).

This may at first seem a rather thin result in the light of the effort that has been put into it, and it is true that it illustrates the difficulty of applying this type of analysis to archaeological remains which have been out of the ground for a long time and in which aDNA is therefore generally poorly preserved and the problems caused by contamination with modern DNA more acute. Nonetheless, we have shown that when hypotheses about kinship can be constructed from existing evidence then the limited aDNA data obtainable from archaeological remains can be used to test those hypotheses and advance understanding. Angel reckoned that of the 21 adults buried in Grave Circle B whose sex could be identified, 16 were male and only 5 female, and he goes on to what one can best describe as a *jeu d'esprit* in speculating about the possible fecundity of Mycenaean rulers and polygamous marriage customs of the period (1973*b*, 389–90). The truth is that by the very nature of this prehistoric and preliterate period we know very little about social relations; that was after all one of the starting-points of this project. So far we have no evidence of brother–sister marriage at this time and place and the discovery of a close kinship between Γ55 and Γ58 does not change that situation significantly. If this was indeed a sibling marriage then it was presumably made possible by Γ58's high birth, but we are left to conjecture whether she was buried in this high-status and male-dominated grave circle because of a marital connection that was linked to her high birth, or because she held a position of authority by right of birth alone.

DNA analysis has thus enabled us to glimpse factors contributing to the organization of the higher echelons of society at the beginning of the Mycenaean age. And for the archaeological scientist this project has pointed the way for future work: the results from Kouphovouno make it very clear that where the samples are taken from freshly excavated bone and under conditions that allow as little contamination as possible, there is indeed much to be learned about the people whose story we are trying to uncover. That, surely, is a great step forward. We like to think that the Mycenaeans—especially Γ58—would have been pleased too.

Manchester Interdisciplinary Biocentre, University of Manchester ABIGAIL S. BOUWMAN
Manchester Interdisciplinary Biocentre, University of Manchester KERI A. BROWN
Manchester Interdisciplinary Biocentre, University of Manchester TERENCE A. BROWN

Manchester Interdisciplinary Biocentre, University of Manchester ELIZABETH R. CHILVERS
College of Medical and Dental Sciences, University of Birmingham ROBERT ARNOTT
The Manchester Museum, University of Manchester A.J.N.W. PRAG

BIBLIOGRAPHY

Angel, J.L. 1966. 'Porotic hyperostosis, anaemias, malarias, and marshes in the prehistoric Eastern Mediterranean', *Science*, 153, 760–3.

—— 1971. *Lerna II: The People* (American School of Classical Studies at Athens, Princeton).

—— 1973*a*. 'Early Neolithic people of Nea Nikomedia', in Schwidetzky, I. (ed.), *Die Anfänge des Neolithikums vom Orient bis Nord Europa* (Cologne), 103–12.

—— 1973*b*. 'Human skeletons from grave circles at Mycenae', in Mylonas 1973, i (Athens), 379–97.

Arnott, R. and Morgan-Forster, A. in press. 'Malaria and the Prehistory of the Aegean', Oxford (BAR IS).

—— and Stuckey, E. 2003. 'Finding the Hydra: the search for malaria in the Prehistoric Aegean', in Polinger Foster, K. and Laffineur, R. (eds), *Metron: Measuring the Aegean Bronze Age. Proceedings of the 9th International Aegean Conference* (Aegeum, 24; Liège and Austin, TX), 209–15.

Bouwman, A.S. and Brown, T.A. 2005. 'The limits of biomolecular palaeopathology: ancient DNA cannot be used to study venereal syphilis', *Journal of Archaeological Science*, 32, 691–702.

—— Chilvers, E.R., Brown, K.A. and Brown, T.A. 2006. 'Identification of the authentic ancient DNA sequence in a human bone contaminated with modern DNA', *American Journal of Physical Anthropology*, 131, 428–31.

—— Brown, K.A., Prag, A.J.N.W. and Brown, T.A. 2008. 'Kinship between burials from Grave Circle B at Mycenae revealed by ancient DNA typing', *Journal of Archaeological Science*, 35, 2580–4.

Brown, T.A. 2001. 'Ancient DNA', in Brothwell, D.R., Pollard, A.M. (eds), *Handbook of Archaeological Sciences* (Chichester), 301–11.

—— Brown, K.A., Flaherty, C.F., Little, L.M. and Prag, A.J.N.W. 2000. 'DNA analysis of bones from Grave Circle B at Mycenae: a first report', *BSA* 95, 115–19.

Cavanagh, W. and Lagia A., forthcoming. 'Burials from Kouphovouno, Sparta, Lakonia', in Philippa-Touchais, A., Touchais, G., Voutsaki, S., and Wright, J. (eds) *Mesohelladika* (Athens).

—— Mee, C., Renard, J. 2007. 'Excavations at Kouphovouno, Laconia: Results from the 2001 and 2002 Seasons', *BSA* 102, 11–101.

Chilvers, E.R. 2004. 'Ancient DNA and palaeopathology: malaria and Ancient Greece' (PhD thesis, University of Manchester Institute of Science and Technology).

—— Bouwman, A.S., Brown, K.A., Arnott R.G., Prag, A.J.N.W. and Brown, T.A. 2008. 'Ancient DNA in human bones from Neolithic and Bronze Age sites in Greece and Crete', *Journal of Archaeological Science*, 35, 2707–14.

Cooper, A. and Poinar, H. 2000. 'Ancient DNA: do it right or not at all', *Science*, 289, 1139.

Evison, M.P. 2001. 'Ancient DNA in Greece: problems and prospects', *Journal of Radioanalytical Chemistry*, 247, 673–8.

—— Fieller, N.J., and Smillie, D.M. 1999. 'Ancient HLA: a preliminary survey', *Ancient Biomolecules*, 3, 1–28.

Gilbert, M.T.P., Bandelt, H.-J., Hofreiter, M. and Barnes, I. 2005. 'Assessing ancient DNA studies', *Trends in Ecology and Evolution*, 20, 541–4.

Lagia, A. 1993. 'Differential diagnosis of the three main types of anaemia (thalassaemia, sickle cell anaemia, iron deficiency anaemia) based on macroscopic and radiographic skeletal characteristics' (MSc dissertation, University of Bradford).

—— Petroutsa E. and Manolis S. 2007. 'Health and diet during the Middle Bronze Age: the site of Kouphovouno', in Mee, C. and Renard J. (eds), *Cooking up the Past: food and culinary practices in the Neolithic and Bronze Age Aegean* (Oxford), 313–28.

Myers, J.W., Myers, E.E. and Cadogan, G. (eds) 1992. *The Aerial Atlas of Ancient Crete* (London).

Mylonas, G.E. 1957. *Ancient Mycenae: The Capital City of Agamemnon* (London).

—— (ed.) 1973. *Ὁ Ταφικὸς Κύκλος Β τῶν Μυκηνῶν*, 2 vols. (Athens).

Nafplioti, A. 2009. 'Mycenae Revisited, Part 2: strontium isotope ratio analysis of the human remains from Grave Circle A', *BSA* 104, 279–91.

O'Donoghue, K., Clapham, A., Evershed, R.P. and Brown, T.A. 1996. 'Remarkable preservation of biomolecules in ancient radish seeds', *Proceedings of the Royal Society of London, series B*, 263, 541–7.

Papakonstantinou, M.Ph. 1999*a*. 'The Grave Circle B of Antron: preliminary report', in Betancourt, P.P., Karageorghis, V., Laffineur, R. and Niemeyer, W.-D. (eds), *Meletemata, Studies in Aegean Archaeology Presented to Malcolm H. Wiener as he Enters his 65th Year* (Aegaeum, 20; Liège and Austin, TX), 626–31.

—— 1999*b*. 'Ο Ταφικός Κύκλος Α της Αντρώνας', in *Πεπραγμένα του Α Διεπιστημονικού Συμποσίου: Η Περιφέρεια του Μυκηναϊκού Κόσμου (Λαμία 1994)* (Lamia), 171–80.

Papazoglou-Manioudaki, L., Nafplioti, A., Musgrave, J.H., Neave, R.A.H. and Prag, A.J.N.W. 2009. 'Mycenae Revisited, Part 1: Stamatakis, Schliemann, and two new faces from Shaft Grave VI', *BSA* 104, 233–77.

Perles, C. 2001. *The Early Neolithic in Greece* (Cambridge).

Prag, A.J.N.W. and Neave, R. 1999. *Making Faces: Using Archaeological and Forensic Evidence*, corr. repr. of 1997 edn. (London).

—— Musgrave, J.H., Neave, R.A.H. and Thimme, D.I. 1995. 'Seven Faces from Grave Circle B at Mycenae', *BSA* 90, 107–36.

Price, T.D. (ed.) 1989. *The Chemistry of Prehistoric Bone* (New York).

Pruvost, M., Schwarz, R., Bessa Correia, V., Champlot, S., Braguier, S., Morel, N., Fernandez-Jalvo, Y., Grange, T. and Geigl, E.-M. 2007. 'Freshly excavated fossil bones are best for amplification of ancient DNA', *Proceedings of the National Academy of Sciences of the United States of America* 104, 739–44.

Rodden, R.J. 1962. 'Excavations at the Early Neolithic Site of Nea Nikomedeia, Greek Macedonia (1961 Season)', *Proceedings of the Prehistoric Society*, 28, 267–88.

Sallares, R. and Gomzi, S. 2001. 'Biomolecular archaeology of malaria', *Ancient Biomolecules*, 3, 195–213.

Sampietro, M.L., Gilbert, M.T.P., Lao, O., Caralelli, D., Lari, M., Bertranpetit, J. and Lalueza-Fox, C. 2006. 'Tracking down human contamination in ancient human teeth', *Molecular and Biological Evolution*, 23, 1801–7.

Smith, C.I., Chamberlain, A.T., Riley, M.S., Stringer, C. and Collins, M.J. 2003. 'The thermal

history of human fossils and the likelihood of successful DNA amplification', *Journal of Human Evolution*, 45, 203–17.

Wardle, K.A. (ed.) 1996. *Nea Nikomedia 1: The Excavation of an Early Neolithic Village in Northern Greece 1961–1964* (BSA supp. vol. 25; London).

Zangger, E. 1991. 'Prehistoric coastal environments in Greece: the vanished landscapes of Dimini Bay and Lake Lerna', *JFA* 18, 1–15.